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The Role of Fungal Competition and Tannin Chemistry in Chestnut and Oak Resistance to *Cryphonectria parasitica*

A Senior Honors Thesis in the Department of Biology, Sweet Briar College

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Abstract

Chestnut blight is an introduced fungus that attacks and kills American chestnuts. It also lives on other chestnut and some oak species but these are not killed by an infection. Many researchers have explored areas such as bark chemistry, genetics, and fungal hypovirulence to try to better understand the varied levels of resistance to chestnut blight seen between tree species. I hypothesized that condensed tannins within each tree species affect competition within the endophytic microbial community and lead to varied levels of resistance. I hypothesized that American chestnut, a tree with a higher level of condensed tannins, has lower microbial competition, making it easier for chestnut blight to establish itself within a tree. I conducted three experiments that compared the diversity of fungi and bacteria found on American chestnut, Chinese chestnut and post oak; quantified the tannin concentrations of bark from the three tree species; and measured the effects of bark extracts from American versus Chinese chestnuts on the growth rates of endophytic fungi. I found a greater frequency of endophytic fungi growing under the bark of Chinese chestnuts and post oaks than under the bark of American chestnuts. Unfortunately, methodological problems did not allow for firm conclusions to be drawn about the tannin content of each species or the effects of tree extracts on fungal growth.

INTRODUCTION

Natural History of the American Chestnut

At the turn of the 20th century twenty five percent of the trees in the Appalachian forests were American chestnuts [*Castanea dentata* (Marshall) Borkhausen, Family Fagaceae] (Griffin 1986). American chestnuts grew up to 100 feet tall and were considered the redwoods of the East coast. The chestnut trees supported large timber and tanning industries because of their size and tannin content. The tannins prevented the American chestnut wood from deteriorating over long periods of time. The nuts from the trees also provided a good food source for both animals and humans because they produced large quantities of nuts yearly, unlike the oaks, which only produce nuts every few years (Newhouse 1990).

Today, the American chestnut exists only as a small understory tree. In 1904 the ascomycete *Cryphonectria parasitica* (Murr.) Barr, or chestnut blight, was first noticed to have entered the United States, from Japan, in New York City (Farlow 1912). Shear and Stevens (1913) suggested that *C. parasitica* was introduced from China because a nearly identical fungus to chestnut blight was found growing on Chinese chestnuts in China. However, the fungus has also been found in Japan (Griffin 1986). Chestnut blight enters a tree's bark through a wound and over time girdles and kills the tree. Over a period of about 50 years chestnut blight killed nearly all of the large American chestnuts throughout the entire Appalachian forests. However, the roots of American chestnuts are not affected by chestnut blight, allowing the trees to resprout from their root collars. Unfortunately the sprouts rarely survive long enough to reproduce before being killed by blight infections.

Chestnut Blight Infection

Spores of *C. parasitica* travel between trees on the wind and by adhering to the feet of birds and insects (Newhouse 1990). When a spore lands on a chestnut tree, germ tubes and hyphae grow into a wound in the tree. Once under the bark the fungus grows into the cambium in the form of a mycelial fan (Griffin 1986). The cambium is a layer of actively dividing cells located under the bark of a tree that produces xylem and phloem, which provide transportation of water and nutrients from the soil to the tree's leaves. The blight excretes unidentified chemicals that kill the cambium. The abundant tannins in the tree are oxidized and used as a food source by the fungus (Rankin 1929, Bramble 1936, Griffin 1982).

After three to four weeks, a canker develops on the tree where the fungus is growing (Griffin 1986). The canker is a tree's reaction to microbial invasion. Cankers vary in size and type. A tree that is effectively fighting an infection will have cankers consisting of areas of bark that are swollen while trees that have had their cambium killed will have cankers with sunken areas of bark. The tree's level of resistance to the fungus determines the severity of the cankers it develops and the length of time the tree can survive an attack. As the fungus grows it eventually girdles the tree by killing a ring of the cambium circumferentially (Newhouse 1990).

Cryphonectria parasitica reproduces both sexually and asexually. When it begins to reproduce, stroma, or fruiting bodies, are seen as tiny red bumps on the canker about 2-4 mm in diameter. The fruiting bodies do not usually form until after the first year of the fungus's growth. When the weather becomes moist, elongated sticky tendrils, similar to curly threads, are produced. These "spore horns" or "cirrhi" contain the unicellular, asexual conidia spores (Alexopoulos 1996, Griffin 1986). The spore horns can occasionally be seen with the naked eye during rainstorms, but frequently they are washed away or splashed onto another tree just as quickly as they are created (Griffin, 1982). They can also adhere to the feet of birds or insects and spread to other trees (Newhouse 1990). After releasing spore horns, the fruiting bodies contain vase-like structures that eject sexual ascospores to travel on the wind to other trees (Newhouse 1990).

Host Range of *C. parasitica*

Not all chestnut species are killed when infected by chestnut blight. In its native Asian range, chestnut blight lives on Chinese and Japanese chestnuts (*Castanea*

mollissima Blume and *C. crenata* Siebold and Zuccarini). These species are resistant to the blight and, though they may develop infections, they usually do not die.

Chestnut blight also entered Europe and attacked European chestnuts (*Castanea sativa* Miller). It was first discovered in Italy in 1938. The fungus spread throughout Italy and many other parts of Europe, though some areas do seem to still be free of blight. The fungus did not spread as rapidly in Europe as in America and the European chestnut population has begun recovering from the invasion.

In addition to chestnut trees, chestnut blight has also been found living on oak trees, particularly the post and scarlet oak (*Quercus stellata* and *Q. palustris, Fagaceae*). These two oak species will develop infections but are also not often killed (Davis and Torsello 1999).

Antimicrobial properties of tannins

Tannins are water soluble, phenolic polymers that can bind and/or precipitate proteins (Temple 1982, Hagerman and Butler 1989). Tannins are one of the most abundant biochemicals in plants (Hernes and Hedgers 2000). Although tannins are involved in basic metabolic reactions, waterproofing, and cell wall strengthening (Hernes and Hedgers 2000, Leegood and Lea 1999), plants predominantly use them as secondary metabolites to ward off herbivores and microorganisms. Tannins will bind to proteins and render this nitrogen source indigestible and reduce enzyme activity (Temple 1982, Leegood and Lea 1999).

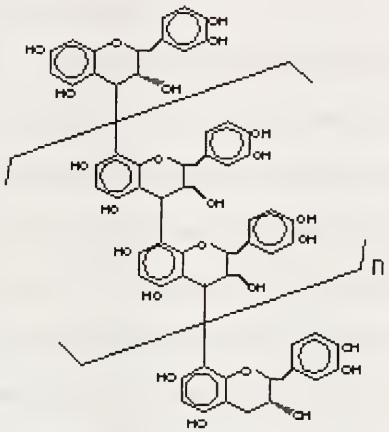
There are two main groups of tannins, condensed and hydrolyzable (see images below). Condensed tannins are made up of oligomers and polymers of 3-ring flavanols (Hernes and Hedger 2000). Hydrolyzable tannins consist of a glucose core and esters of

hydrolyzable or condensed tannins as the only carbon source. Nine of the bacteria grew with hydrolyzable tannins and only four grew with condensed tannins.

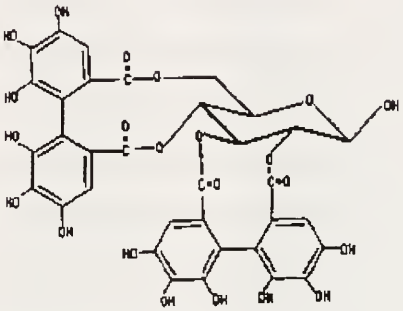
Bhat et al (1998) also list twenty-nine fungi that produce tannase, many of which are in the genera *Asperigillus and Penicillium.* Eight of these twenty-nine fungi are listed as good or best producers of tannase compared to other fungi. Lewis and Starkey (1969) surveyed the ability of soil fungi to decompose hydrolyzable and condensed tannins. They isolated filamentous fungi from a sandy soil and then grew the fungi on medium containing either hydrolyzable tannins or condensed tannins. They did not count the number of isolates found on each medium type but they made the qualitative observation that more fungi grew on the medium containing hydrolyzable tannins than the condensed tannins.

Tannins are common in the plant kingdom but the concentrations of tannins vary among plant species and among the organs and tissues of a plant in which it is found (Bhat et al1998). Tannin concentration can also vary seasonally within a tree or environmentally within a species. Feeny and Bostock (1968) found that the total tannin content in the leaves of a single *Quercus robur* increased from April to September. Reed and McCarthy (1996) studied the tannin content of chestnut oak (*Quercus prinus* L.) and black oak (*Quercus velutina* L.) growing on north vs. south- facing slopes. They found that chestnut oaks had larger amounts of tannins in their leaves on north-facing slopes than on south-facing slopes but the tannin content of the black oak was not slope dependent.

gallic acid at several alcohol positions. When condensed tannins are broken apart enzymatically, the corresponding subunits of flavanol are still toxic; they still have protein-binding properties. In contrast, when hydrolyzable tannins are biodegraded, the resulting sugar and gallic acid moieties are no longer toxic (Hemmingway and Branham 1992).



Condensed Tannin



Hydrolyzable Tannin

There are more microorganisms (including fungi, yeasts, and bacteria) that are capable of breaking down hydrolysable tannins than condensed tannins. Most of these organisms that tolerate or use tannins contain tannase, an enzyme that breaks down hydrolyzable tannins through oxidation (Farris 1992,1994). In their review paper, list seven bacteria that produce tannase, all of which are moderate to good producers. Dischamps et al (1980, cited in Bhat et al (1998)) investigated at the growth of fifteen bacteria in the genera *Bacillus, Staphylococcus*, and *Kebsiella* on medium with either

Resistance to Chestnut Blight Disease

Since *C. parasitica* first entered the United States scientists have been trying to discover what factor(s) contribute to some chestnut trees' ability to resist a blight infection (Griffin 1986, Anagnostakis 1987, Kubisiak et al 1997). There are three main areas of research.

Host Tree Genetics

Clapper (1952) suggested that two genes control resistance. He performed an experiment in which he crossed American chestnuts with Chinese chestnuts and then backcrossed the hybrids to Chinese chestnuts. The ratio of three highly resistant trees to one low resistance tree in the backcross generation was compatible with a two-gene model. There is some evidence from Clapper's crosses that the resistance genes show incomplete dominance because the Chinese x American trees showed an intermediate level of resistance between that of the Chinese chestnut and the American chestnut (Griffin 1986).

Some researchers think that more than two genes contribute to resistance. Japanese chestnuts are often not as winter-hardy as other species and this reduces their ability to fight off *C.* parasitica infection. Environmental factors seem to contribute to the Japanese chestnut's ability to fight off the infection. If conditions are more favorable to the Japanese chestnut's growth, then it is better able to ward off the blight. When the tree is required to also contend with environmental conditions that are stressful, it is unable to prevent the spread of the chestnut blight infections as well. Genes that affect the tree's ability to deal with the stress of its environment may also contribute to the tree's level of resistance (Griffin 1986).

Clapper's results led to the development of a backcross breeding program of American chestnut and Chinese chestnuts to develop trees that are 15/16th American chestnut and 1/16th Chinese chestnut. The Wagner Research Farm in Meadowview, Virginia has begun breeding 15/16th American chestnut trees. The Lesesne State Forest in Nelson County, Virginia has the largest planting of 50% American chestnut trees and is also backcrossing trees for resistance. However, they are farther behind the Meadowview farm and only have successful plantings of the first backcross trees.

Hypovirulence

A double-stranded RNA virus can infect *C. parasitica* and render it less virulent, or hypovirulent (Alexopoulos et al. 1996). In Europe, hypovirulence has stopped the progression of chestnut blight in the European chestnut (*Castanea sativa)* (Griffin 2000). However, the virus is only spread through hyphal fusion of the fungus and in a percentage of the asexual spores, and is not present in sexual spores. (Griffin 2000). Research is still being carried out to better understand this virus and develop methods to encourage its spread in the United States (Fulbright 1984, Enebak et al 1994, Peever et al 1997, Anagnostakis et al. 1998, Chen et al 2000).

Tannins

Several scientists have hypothesized that tannins may be a part of a chestnut tree's resistance to blight (Anagnostakis 1992, Farias 1992, Griffin 1986). The high concentrations of tannins in American chestnuts reportedly allow them to effectively ward off most fungal infections (Anagnostakis 1992). *C. parasitica*, however, produces tannase that is capable of breaking down hydrolyzable tannins, which allows *C. parasitica* to use the subunits of tannins as a food source (Farris et al 1992, Griffin et al.

1982, Bramble 1936). Previous research has shown that *C. parasitica* will grow faster when grown with tannins than will other related fungal species (Cook and Wilson 1915; the article did not specify whether the tannins were hydrolyzable or condensed). Bramble (1936) suggested that the increased growth rate of blight, when exposed to tannins, helps the fungus to grow faster than the American chestnut's efforts to block the attack with callus, or thickened bark, formations.

Variation in concentrations of tannins among chestnut tree species has been explored as a possible explanation for their differing resistance levels. Chinese chestnut has been reported to contain only hydrolyzable tannins in its bark, but American chestnut contains both hydrolyzable and condensed tannins. The European chestnut has both types of tannins, but it has a lower concentration of condensed tannins than the American chestnut (Griffin 1986). I have not found literature citations that report the tannin content of oak bark, but both types of tannins exist, with seasonal variation, in oak leaves. In early spring, the leaves of *Quercus robur* contain mostly hydrolyzable tannins, but by early summer condensed tannins begin to appear (Feeny and Bostock 1968).

Nienstaedt (1952) studied the growth of *C. parasitica* on medium with tannin extracts from the Japanese, Chinese, and American chestnut trees. He found that the Japanese and Chinese chestnut extracts inhibited the growth of *C. parasitica* compared to its growth on medium with American chestnut extract. However, Nienstaedt used water to extract his tannin samples and did not buffer his growth medium. Hagerman (1988) tested several extraction methods and determined that the best solvent for extracting tannins is 70% acetone. When Anagnostakis (1992) used 70% acetone for extractions she was unable to find a difference in growth between chestnut blight fungus grown with

tannins from Chinese chestnuts and with tannins from American chestnuts in a buffered medium. Anagnostakis concluded that the tannin differences in each chestnut species cannot explain the varied levels of resistance in each tree species.

Fungal Diversity

There are many microorganisms growing on, under, and in the bark and leaves of trees. Kowalski and Kehr (1996) presented a study of the colonization by endophytic fungi in the living and dead branches of 11 European tree species. Nearly 100% of the peridermal bark samples and 6-43% (species dependent) of subperidermal bark samples of each tree species were colonized by endophytic fungi. The periderm is the outer layer of bark cells and the subperiderm is the inner layer. Kowalski and Kehr (1996) suggested that some of these fungi assist in branch pruning once a branch has died and that some of the fungi are adapted to only exist endophytically. In one of their examples, 60 species of fungi were found under dead bark of the *Quercus robur* (English Oak), 10 species within living bark tissue, and 4 species in the wood.

Müller and Hallaksela (2000) surveyed one entire healthy Norwegian spruce (*Picea abies),* growing in a Norway spruce, Scots pine (*Pinus sylvestris)*, and pubescent birch (*Betula pubesces)* mixed forest, for the filamentous fungi, yeast and bacteria growing on and within the needles, branches and trunk of the tree. They found a high diversity of microbes, with the microflora dominated by fungi (99 species) and with very few bacteria present (8 kinds). On nonsterilized needle surfaces they found 84 different kinds of epiphytic fungi. Although more fungi were found on external surfaces than internally, they did find forty-eight different kinds endophytic fungi in the tree (needles,

twigs, bark, and wood). Muller and Hallaksela also showed that more fungi were growing endophytically on the north side of the tree than the south.

Johnson and Whitney (1988) surveyed the endophytic fungi found in 840 surface sterilized needles of a balsam fir in a spruce budworm deforestation site in New Brunswick, Canada. They found 771 fungal isolates but did not specify how many different species were represented. They also showed that the number of fungal isolates found increased as the height of the sample from the ground increased. Johnson and Whitney also investigated the compass direction diversity on the tree but found no significant difference in diversity on the north versus south side of the tree.

Thesis

I propose that resistance in chestnut and oak trees to chestnut blight is due to the degree of fungal competition present under the bark of each tree species and that this competition is influenced by the concentration of condensed tannins present in a tree's bark. Though both types of tannins are toxic to microorganisms, condensed tannins are toxic to more organisms because few organisms can alter condensed tannins into a nontoxic form. The absence of condensed tannins in the Chinese chestnut may allow more fungi to grow and coexist under its bark than under the bark of the American chestnut, which contains both hydrolyzable and condensed tannins. If the Chinese chestnut is host to a variety of fungi then chestnut blight fungus may be unable to compete well on the tree; it may never be able to grow rapidly enough to cause serious damage to the tree. If the condensed tannins in the American chestnut decrease the number of fungi that can grow on it, then *C. parasitica* will have less competition and can rapidly grow unhindered.

In Experiment 1 I looked at the frequency of microorganisms and the diversity of fungi within American chestnut, Chinese chestnut, and post oak (*Quercus stellata* Wangenh) trees. Post oaks were sampled because they are attacked by chestnut blight disease but usually survive (Griffin 1986). Experiment 2 was designed to compare the total tannin and condensed tannin content in bark extracts from each tree species. Experiment 3 then tested the fungi from experiment 1 with the extracts from experiment 2 in order to determine if the tannins of Chinese versus American chestnut extracts would affect the growth rates of selected fungi from each tree species.

EXPERIMENT 1. FUNGUS SURVEY

I expected to find a higher diversity and/or abundance of fungi (filamentous fungi) under the bark of Chinese chestnut and post oak trees than under the bark of American chestnuts. I sampled the bark of the American chestnut, Chinese chestnut, and post oak trees to determine how many fungi inhabit them.

Tree Sources

Five trees from each species were sampled. I collected samples from three Chinese chestnuts (DBH:43.8cm, 57.2cm, 69.2cm) in the Meta Glass parking lot on Sweet Briar campus and two (DBH: 20.0cm, 27.2cm) from Fresh Water Cove Lane in Nelson County, Virginia. The Sweet Briar trees were growing in an open area and the Nelson County trees were growing just within a forest. Four American chestnut trees (DBH: 5.5cm, 6.6cm, 9.6cm, 12.0cm) were sampled in the Lesesne State forest in Nelson County and one tree (DBH not recorded) came from another location in Nelson County. The Lesesne State Forest trees were growing in a backcross orchard (but were pure

American chestnut) and the Nelson county tree was growing under a power line in a wooded area. All American chestnut trees were infected with chestnut blight and had many new sprouts. The branch samples came from the main tree and not the sprouts; the branches collected also did not have obvious cankers. All but one were collected between December 16, 2002 and January 3, 2003. The final American chestnut sample was collected on January 25, 2003. Three post oak (DBH: 11.3cm, 34.7cm, 36.0cm) trees came from Blue Ridge Lane in Amherst, VA, one (DBH: 33.7cm) came from Elijah Road on Sweet Briar campus, and one (DBH: 67cm) came from the entrance road on Sweet Briar campus. Two of the Amherst trees were growing on the edge of a small wooded area. The third Amherst tree was growing in an open area. This tree had many unidentified cankers on its trunk but the samples came from healthy branches.

Collection Methods

To collect the fungi from trees I cut off small branches (about 3-5cm in diameter at base) and took them into the lab. In order to accurately survey the tree, I insured that the collected branches were well spaced around the tree. This distancing was accomplished by collecting one sample from, roughly, each of the four compass directions. Oak tree number one only has three samples (north, east, and south-east) because only three sides of the tree had branches within reach of my saws. The height of the branches collected ranged from 1.5 to 7.6m depending on the size of the tree and location of the branches.

The fungus samples were collected from branches and not the trunk because they could be taken into the lab and their removal did not cause as much damage to the trees.

In the lab, within 24 hours of collecting branches, I removed small wedges of bark and placed them in a fungal growth medium. I surface sterilized an area of bark about 10cm in length and 3cm in width with 70% ethanol. I used a flame-sterilized razor to cut out a thin wedge of wood (about 3mm in width and 5mm in length), including the bark, and placed the wedge into potato dextrose agar (Difco and Carolina Biological; brands were switched from Carolina to Difco midway through experiment). The plates used for the agar were 9cm in diameter. Two wedges collected within 5cm of each other from each branch were placed in one agar dish. Eight wedges were collected per tree. The wedge samples were incubated at 32°C. After the fungi under the bark began to grow onto the agar, I isolated each type by using a sterilized 5mm size cork borer to transfer a plug of the fungus and agar to another sterile agar dish. Most fungi were visible with the naked eye within two or three days. However, because the samples were collected during the winter the wedges were incubated for at least a week in order to allow dormant fungi to begin growing.

The growth rate of each fungus sample was determined by transferring a 5mm plug to the center of a new plate and then measuring the diameter of the fungus daily over a seven day period. The diameter was determined by taking two measurements perpendicular to each other with a cm ruler and then recording the average of the two.

To document the diversity of the filamentous fungi each isolate was photographed 3-4 days after transfer to a new plate and 25+ days after transfer. The photographs were taken with a Nikon 8008 with a 105mm macro lens and ring flash using Kodachrome or Fujichrome 100 film. The visual comparison was completed by using 4x6 inch prints of

each sample. Samples that had similar growth rates and patterns, structures, and color were considered to be the same species..

All statistical analyses were done using StatView 5.0.1 (SAS Institute 1998).

Results

Abundance of all microbial groups.

The data collected were the number of filamentous fungi, bacterial and yeast colonies isolated from each tree (Table 1). The five Chinese chestnut trees produced a total of 71 colonies from the 40 bark wedges (8 wedges per tree), 57 of which were fungi and 14 of which were yeast and bacteria. The post oak had 73 microbial colonies, which included 63 fungi and 10 yeast and bacteria. The American chestnut contained fewer microorganisms than the other two tree species. It had 41 fungi and 3 yeast and bacteria for a total of 44 colonies. A one factor ANOVA showed that the abundance of microorganisms differed among tree species ($F_{2,12} = 5.623$, $p = 0.019$). The American chestnut trees had significantly fewer microorganisms than the post oak and Chinese chestnuts (Fisher's test, $p = 0.0035$ and 0.0022). There was no significant difference in the number of microorganisms growing on the post oaks and Chinese chestnuts (Fisher's test, $p = 0.7932$).

Table 1: The abundance of fungi and yeast and bacteria found on each tree sampled. Data are the total number of isolates grown from 8 bark chips per tree.

Tree	Total	Fungi	Yeast & Bacteria
Chinese Chestnut Trees			
1	13	9	4
2	11	11	0
3	16	13	3
4	17	13	4
5	14	11	3
total	71	57	14
Post Oak Trees			
1*	11	10	1
2	12	9	3
3	18	17	1
4	17	14	3
5	15	13	2
total	73	63	10
American Chestnut Trees			
1	8	8	0
2	10	9	1
3	9	8	1
4	7	7	0
5	10	9	1
total	44	41	3

*Post oak tree number 1 only had 6 woodchips sampled.

The abundance of fungi also differed among tree species (Table 1; one factor ANOVA, $F_{2,12} = 5.623$, $p = 0.0189$). The American chestnut has significantly fewer fungi than the Chinese chestnut and post oak (Fisher's test, $p = 0.0361$ and 0.0070). There is also a relationship between bacteria and yeast abundance and the tree species (One factor ANOVA, $F_{2,12} = 4.650$, $p = 0.0320$). The American chestnut has significantly fewer isolates of bacteria and yeast than the Chinese chestnut (Fisher's test, $p = 0.0108$) but not fewer than the post oak (Fisher's test, $p = 0.0793$). The Chinese chestnuts and post oaks do not have a significantly different abundance of bacteria (Fisher's test, $p = 0.2948$).

Fungal diversity.

Table 2 lists the number of different types of fungi found on each tree. The Chinese chestnut had an average of 10.0 different fungi per tree, the post oak had an average of 11.2 different molds per tree, and the American chestnut had an average of 7.0 different fungi per tree. This difference approaches statistical significance (One factor ANOVA, $F_{2,12} = 3.656$, $p = 0.0576$).

Table 2: The number of different fungi grown from eight woodchips from each tree sampled

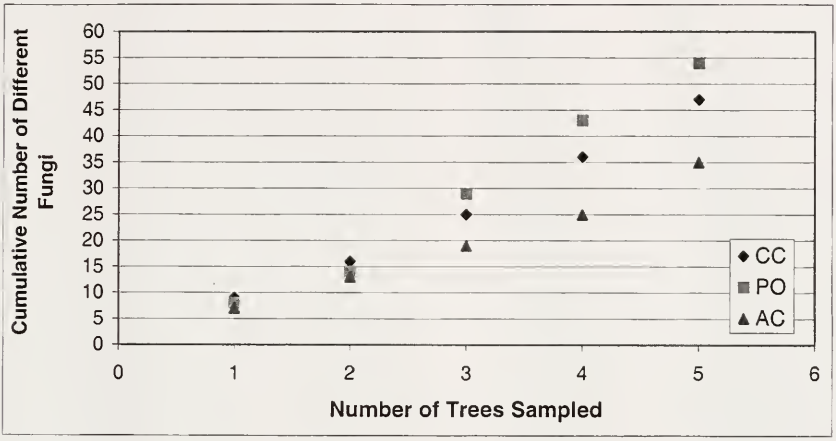
Tree	# Different Fungi	Lost**
Chinese Chestnut Trees		
1	9	0
2	8	4
3	11	0
4	11	0
5	11	0
Average (s.d.)	10.0 (1.4)	
Post Oak Trees		
1	9	0
2	6	0
3	16	1
4	14	1
5	11	0
Average (s.d.)	11.2 (4.0)	
American Chestnut Trees		
1	7	2
2	6	1
3	7	1
4	6	0
5	9	0
Average (s.d.)	7.0 (1.2)	

**The lost fungi were those that stopped growing and were unable to be maintained

Table 2 indicates the diversity of fungi on individual trees, but does not indicate the total number of different fungi found on each tree species. Figure 1 is the cumulative diversity of fungi isolated from each tree species as the number of trees sampled

increased. The fungal diversity increased at a greater rate on the post oak than the Chinese chestnut. The American chestnut had a slope lower than the other two tree species. A total of 54 different fungi were isolated from post oak, 47 from Chinese chestnut, and 35 from American chestnut.

Figure 1: The cumulative number of different fungi found as the number of trees sampled increases.

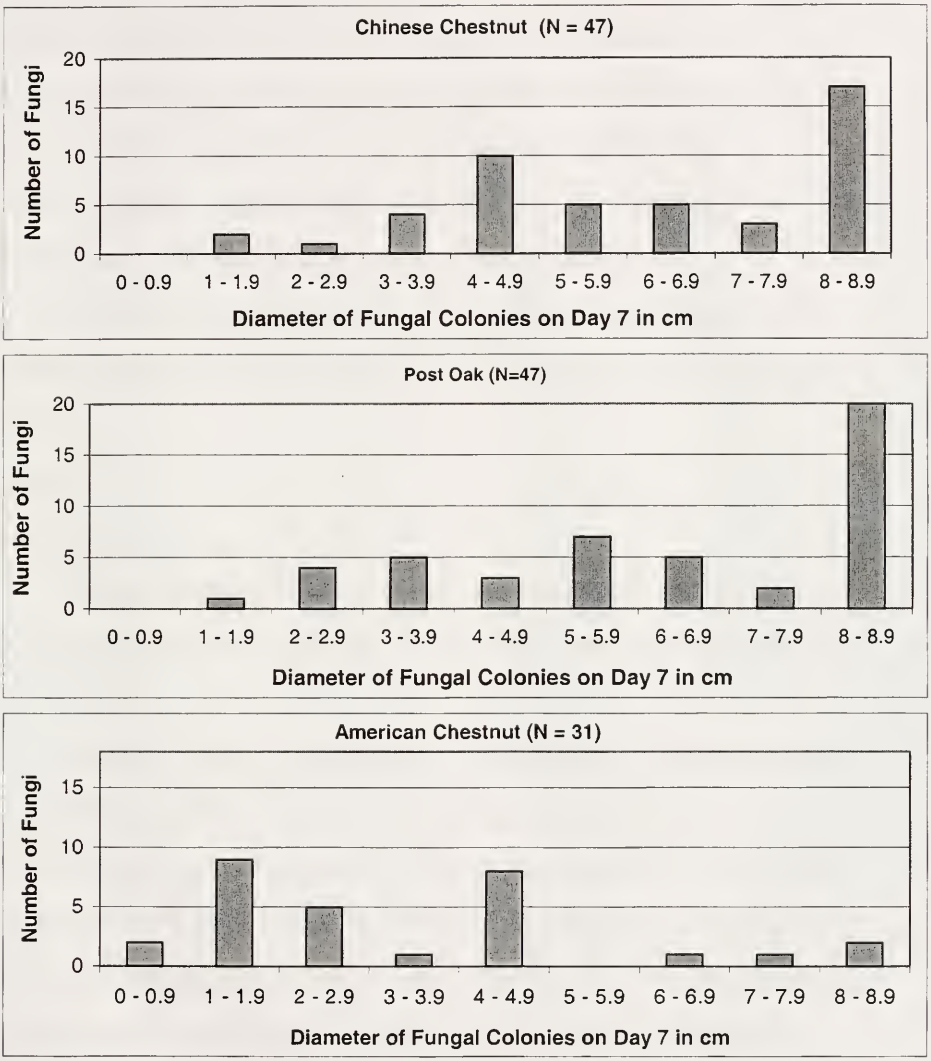


Fungal growth rates.

The fungi isolated from the American chestnut had slower growth rates than the fungi isolated from the Chinese chestnut and post oak. The histograms in Figure 2 show the fungal growth rates for the colonies isolated from each tree species. When more than one isolate of a single fungus species was obtained the average of their growth rates was used in the figure and analysis. From post oak, 37 out of 47 fungi were ≥4cm in diameter after seven days of growth (78.7%) and from Chinese chestnuts, 40 out of 47 (85.1%) were ≥ 4cm. In contrast, American chestnut had only 12 out of 31 total fungi (38.7%) ≥4cm in diameter, and it was the only tree species with any fungal isolates growing less than 1.0 cm in 7 days. This difference in growth rates of fungi from each tree is

statistically significant (One factor ANOVA, $F_{2,21} = 21.977$, $p=<0.001$). The growth rates of the fungi from the American chestnuts are significantly lower than the growth rates of the fungi from the Chinese chestnuts (Fisher's test, $p = <0.001$) and the post oaks (Fisher's test, $p = <0.001$).

Figure 2: Growth rates of fungi obtained from each tree species when grown on Difco potato dextrose agar. When more than one isolate of a single fungus species was found the average of the growth rates was used in the figure.



EXPERIMENT 2. TANNIN CONCENTRATIONS

My hypothesis not only depends on the number of fungi growing on each species of tree, but also on the concentration of condensed tannins in each tree species. American Chestnuts have been reported to have condensed and hydrolyzable tannins while the Chinese chestnut only have hydrolyzable tannins (Griffin 1986) If my prediction is correct, I would expect that if oak trees have condensed tannins in their bark they will be at a lower concentration than in American chestnuts.

I measured the condensed and hydrolyzable tannin concentrations in the American chestnut, the Chinese chestnut, and the post oak using methods from Hagerman (2002).

Methods

Bark extracts.

With one exception, the north and south branch samples collected for the survey experiment were used for this experiment. For post oak 1 I used the SE branch instead of a South branch.

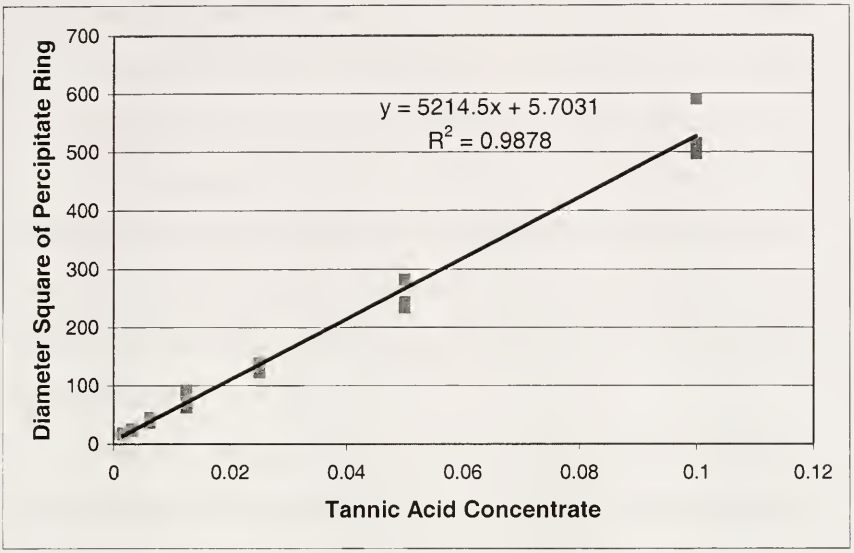
To extract the tannins I scraped off 10-12 grams of bark and cambium from each branch and then ground it into a fine powder with an electric coffee grinder. Once ground, 8.0 grams of bark was placed in a centrifuge tube with 20.0ml of 70% acetone and sonicated at 4°C for 30 minutes. The bark was then centrifuged at 2500xg and the acetone was evaporated off. This process was repeated four more times. [In a pilot study I repeated the process 8 times and determined, using the radial diffusion methods later described, that the tannins were only located in the first 3-4 extracts.] Once extracted the crude extracts were stored in 150ml Wheaton jars at 4°C in the dark.

Determination of total tannin concentration.

After the crude extraction was complete, I determined the concentration of total tannins by using a radial diffusion assay. When a solution containing tannins is placed into a well that is cut into agar containing bovine albumin, the tannins diffuse into the agar until they meet free proteins and precipitate. A white ring will form around the well where the protein has been bound by tannins.

The plates were made of 10% agarose and 1% Bovine Serum Albumin (Fraction V powder, Sigma) in buffer (0.05M acetate containing 60μM ascorbic acid; pH 5.0). The wells were 3.0mm in diameter. Each plate contained 8.0μl of extract per well and was incubated for four days at 32°C. The ring diameter was measured with calipers, to the nearest 0.05mm, while observing the rings under a dissecting microscope. The square of the diameter of the ring is proportional to the concentration of tannin in the sample. A standard curve was created with tannic acid (Sigma); concentrations of tannins are therefore reported as tannic acid equivalents (Figure 3)

Figure 3: Standard curve of known concentrations of tannic acid in standard radial diffusion plates.



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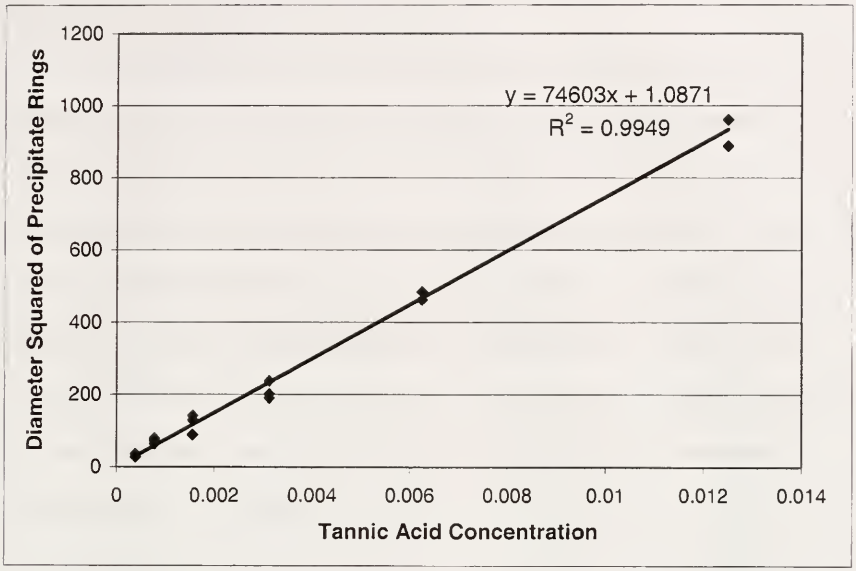
Determination of condensed tannin concentrations.

To assay for the condensed tannins the hydrolyzable tannins were degraded before running a second radial diffusion assay. To degrade the hydrolyzable tannins, 0.25 µl of extract was mixed with 300 µl of 2M hydroxylamine hydrochloride ($NH_2OH{\cdot}HCL$) in ethanol:water (48/52, v/v). Because I expected the condensed tannin rings to be too small to accurately read on standard radial diffusion plates, I made higher sensitivity plates by decreasing the amount of albumin used from 1% to 0.1%. I also increased the amount of extract placed in the well to 16µl. Because the increased sensitivity plates created rings that were faint and difficult to see I had to stain rings to more accurately measure them. To stain the rings the agar was first removed from the plate and placed in a small specimen dish. I was able to easily remove the agar gels by

first lining the plates with plastic wrap before pouring agar into the plates. After removal each agar sample was washed, by shaking, with 40ml of 0.3 NaCl for 20min and then rinsed twice with 10ml of saline. The washing and rinsing process was repeated five times. The NaCl washes remove all excess proteins from the gels so that only the proteins bound to tannins will be stained. After the washing process was complete the gels were stained with 20ml of a Prussian blue reagent until the rings were visible (3-5 minutes). The reagent was made with equal volumes of 0.10M $FeNH_4(SO_4)_2$ in 0.10M HCl and 0.008M $K_3Fe(CN)_6$. After staining the gels were rinsed with 0.1M HCl before measuring the rings.

I produced a new standard curve with tannic acid using the high sensitivity, stained plates (Figure 4), and determined the condensed tannin concentration using this standard curve and the sizes of the rings from the extracts.

Figure 4: Standard curve of known concentrations of tannic acid in increased sensitivity radial diffusion plates.



Once the concentration has been determined, the grams of tannin present can be calculated by multiplying the concentration by the total volume of extract.

Results

The American chestnut trees contain an average of 0.18 grams of total tannins per 8 gram sample, the Chinese chestnut trees contain an average of 0.26 grams of total tannins, and the post oak trees contain an average of 0.21 grams of total tannins (Table 3). These values are not significantly different (One factor ANOVA, $F_{2,12} = 1.631$, $p = 0.2362$).

By inspection, there was also no significant difference in the condensed tannin content among the tree species. The average total condensed tannin content in the

American chestnuts is 0.11 grams. Both the Chinese chestnut and the post oak trees have an average of 0.10 grams of condensed tannins.

An average of 61.8% of the tannins in the American chestnut trees were condensed tannins; the Chinese chestnut trees had an average of 36.8% condensed tannins and the post oak trees had an average of 49.3% condensed tannins (Table 3). There is a significant relationship between the percent condensed tannins and the tree species tested (One factor ANOVA, $F_{2,12} = 4.338$, $p = 0.0408$). The percent condensed tannins in the American chestnut is significantly different from the Chinese chestnut (Fisher's test, $p = 0.0178$) but not from the post oak (Fisher's test, $p=0.538$). The percent of condensed tannins in the Chinese chestnut and post oak are also not significantly different (Fisher's test, $p=0.6501$)

Table 3: Tannin content of bark samples. Data given as average grams of total tannins and condensed tannins found in an eight grams wet sample of bark from each tree; measured as tannic acid equivalents. % condensed tannins = grams condensed tannins/grams total tannins x 100.

Tree	Total tannin (g/8 g sample)	Total condensed tannins (g/8g sample)	% condensed tannins
Chinese Chestnut Trees			
1	0.24	0.07	29.2
2	0.25	0.14	57.1
3	0.28	0.09	32.1
4	0.31	0.08	25.8
5	0.23	0.10	43.5
Average (s.d.)	0.26 (0.03)	0.10 (0.03)	37
Post Oak Trees			
1	0.04	*	*
2	0.19	0.09	47.4
3	0.34	0.11	32.4
4	0.28	0.13	46.4
5	0.19	0.08	42.1
Average (s.d.)	0.21 (0.11)	0.10(0.02)	49
American Chestnut Trees			
1	0.20	0.08	40.0
2	0.24	0.15	62.5
3	0.20	0.13	65.0
4	0.14	0.13	92.9
5	0.11	0.06	54.6
Average (s.d.)	0.18 (0.05)	0.11 (0.03)	62

* not available

EXPERIMENT 3. GROWTH RATES OF FUNGI EXPOSED TO AMERICAN AND CHINESE CHESTNUT EXTRACTS

If tannins are affecting the fungal competition levels in each tree species, then the fungi that do not naturally grow with condensed tannins should have a slower growth rate when grown with condensed tannins than when grown without condensed tannins. A decreased growth rate in the endophytic fungi should lower the degree of competition the chestnut blight is up against when it enters the tree. A subset of the fungi isolated in

experiment 1 from American and Chinese chestnuts and a *C. parasitica* sample were grown on potato dextrose agar and on potato dextrose agar supplemented with extracts from American chestnut and Chinese chestnut bark.

Methods

From the isolates obtained in experiment 1, five fungi from the American chestnut trees and five fungi from the Chinese chestnuts were selected. To make the selection, the fungi from each category were divided into growth categories (extra slow, slow, medium, and fast) based on their diameter after seven days of growth. The slow and fast fungi were then lumped together and five of them were randomly selected for testing. One virulent chestnut blight fungus, PS-713, was provided by Dr. Gary Griffin of the Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech in the summer of 2001.

To ensure that the tannin concentration the fungi were exposed to in this experiment was the same as that found in trees in the field, the extract from 8 grams of fresh bark was used to make 8 grams of wet agar. From Experiment 2 I had approximately 220ml of extract from 80g bark for each tree species. This was enough to make only 9-11 plates per extract, because a single agar plate with 7.5ml agar weighed 7.04g.

In order to keep the concentrations of tannins added to the agar equivalent to the concentrations within bark, the active ingredients from the 220ml of extract needed to be incorporated into 75ml of agar, thus requiring the extract to be concentrated. First a Büchi R-114 rotary evaporator was used to remove the acetone from each bark extract. This process was completed at 20°C. To remove some of the remaining water, the extracts

were then lyophilized for three days using a Flexi-Dry MP FTS System. After these procedures there was approximately 18ml of highly concentrated Chinese chestnut extract and 25ml of highly concentrated American chestnut extract. Though the volumes were not equal, the tannin content of the solutions came from an equal weight of bark.

In pilot test plates I discovered that double strength extract added to 4% agar made agar that would not completely solidify and double strength extract plus 8% agar would solidify too fast to accurately pour plates before solidifying. I therefore decided to make medium with a final agar concentration of 3%. To make 3% agar with tannins, extract containing concentrated bark tannins (twice the final desired concentration) was mixed with MES sodium salt buffer (Sigma) (to make a 1.0M solution) and double strength Potato Dextrose Broth. The solution was filter sterilized with a 115ml Nalgene disposable filter with 0.2µm pores and mixed with an equal volume of 6% agar. Unfortunately, the 6% agar still began to solidify in the jars so I was unable to use pipettes to measure equal amounts of agar into each plate.

A 5mm plug of each isolate was placed on one plate each containing American chestnut extract, Chinese chestnut extract, and agar without bark extract. Two diameters of the fungi were then measured and averaged daily over a one-week period. Because the agar was too dark to easily see through from the bottom of the plate the fungi were measured through the lids. To be sure the same place was measured on the fungi each day two lines were drawn perpendicular to each other on the plate lids and the lids were held in place with parafilm.

The growth rate of each isolate grown with its host extract was compared to its growth rate with the alternate extract.

Results

The results of this experiment may not be accurate due to several problems that arose throughout the experimental time. (a) Due to an autoclave malfunction the control plates and several of the extract plates became contaminated. (b) Because the 6% agar solidified rather rapidly, the agar had to be quickly hand poured instead of measured equally into each plate. I also had difficulty pouring enough plates so the agar from a few of the complete plates was cut in half and divided into two plates with a sterile spatula. The agar was also very lumpy and had many clumps in which the fungi would have to be growing upwards instead of horizontally, thus affecting the measured growth rate. (c) While filtering the Chinese chestnut extract, water back washed through the vacuum tube into the collection container, decreasing the initial concentration of the extract. The new volume of Chinese chestnut extract was not measured in order to prevent contamination of the sterile solution, but I estimate that the extract was diluted 10-15%, compared to the American chestnut extract.

Table 4 shows the results of the fungal growth rate experiment. The upper part of the graph shows six pairs of plates that could be directly compared because they did not have contamination and the plates from each extract had the same area (full or half full) of agar. The bottom half of the table shows the plates that could not be used for comparison. The chestnut blight fungus, three American chestnut isolates and one Chinese chestnut isolate grew faster on the Chinese chestnut agar than on the American chestnut agar. The sixth sample did not grow during the eight day period. No fungus grew more quickly on the American chestnut agar than on the Chinese chestnut agar.

Table 4: Growth of fungi on agar supplemented with American chestnut or Chinese chestnut bark extracts. The data are given as the diameter of each fungus after eight days of growth on each medium type. The initial diameter of the isolates on Day 0 was 0.5cm. The top six samples are fungi that can be directly compared. The highlighted cells represent those plates that are contaminated. AC = American chestnut CC = Chinese chestnut

Sample	A.C. extract (Diameter, cm)	C.C. extract (Diameter, cm)	A.C >/</= C.C
PS-713 (*C. parasitica*)	1.15	1.45	<
AC 36-14Na	0.75	1.20	<
AC 36-7Nb3	4.20	9.00	<
AC 34-3 a2	2.50	3.00	<
CC 4Wb	4.70	7.00	<
CC 1Wb1	0.50	0.50	No Growth
AC 35-2Wb	0.60	0.60	
AC 34-2Na	0.75	1.20*	
CC 2Wa2	0.60	0.60	
CC Sb2	0.75*	1.00*	
CC 5Eb3	1.55	0.75	

*Due to trouble pouring the medium these plates were only half full.

DISCUSSION

Evidence supporting the original hypothesis

I hypothesized that varied levels of resistance among chestnut and oak tree species were due to varied levels of competition among their endophytic fungi, and that the competition was affected by condensed tannin concentrations.

I first predicted that the American chestnut trees would have a lower frequency and fewer types of microorganisms growing under the bark than the Chinese chestnut and post oak trees. Experiment 1 supported both of these predictions: a significantly lower number of fungi were isolated from under the bark of the American chestnuts than from under the bark of Chinese chestnuts or post oaks. American chestnuts also had fewer types of fungi among the isolates, although this trend was not statistically significant.

The cumulative frequency of different fungi on each tree species also supports my hypothesis (Figure 1). The data show that the number of types of fungi found on the Chinese chestnuts and post oak trees increased at a faster rate than on the American chestnut trees as the number of trees sampled in each species increased from 1 to 5. *C. parasitica* should face a lower level of competition against the lower frequency of fungi found on the American chestnut tree than the higher frequency of fungi found on the Chinese chestnut and post oak trees.

I also found an interesting trend in the growth rates of the fungal isolates. In the absence of tannins, the fungi from the American chestnuts grew significantly slower than the fungi from the Chinese chestnuts and post oaks (Figure 2). If this trend were to hold up when the fungi were grown on their host tree, then the faster growing fungi on the Chinese chestnut and post oak trees may also contribute to these trees' resistance to chestnut blight disease. It may be easier for *C. parasitica* to compete with slow growing fungi on the American chestnut and establish a firm hold under the tree's bark than to compete against the fast growing fungi in the Chinese chestnuts and post oak trees. It may also be possible that there are slow growers on the Chinese chestnuts and post oaks but, because of the large number of fast growers, I was unable to sample the slow growers. The fast growers would have grown out from under the bark faster than the slow growers and established themselves on the agar before a slow grower, preventing me from isolating the slower growing fungi.

I also predicted (experiment 2) that there would be higher levels of condensed tannins found in the American chestnut trees than in the Chinese chestnut and post oak trees. In fact, the concentration of condensed tannins in bark samples was the same for

each tree species. My results did show a difference in the percent of total tannins that are condensed tannins, with American chestnuts having a higher percentage of condensed tannins than Chinese chestnut and post oak. If the results are correct, than it would mean the total tannins or condensed tannins may not contribute to the differences in fungal frequency and diversity previously shown. However, it has been previously reported that Chinese chestnuts should not have condensed tannins (Griffin 1986). A possible explanation for the discrepancy between my results and the literature is in the digestion methods. As a control, I digested a known concentration of tannic acid, which is a hydrolyzable tannin. After the digestion, the tannic acid sample should have produced no ring in the radial diffusion assay, but in fact a small, faint ring was visible on the high sensitivity plates around each of the three wells containing the digested tannic acid. I followed Hagerman's (2002) methods but some part of the procedure may not have gone as expected: the hydroxylamine hydrochloride, for example, was of considerable age and unknown purity.

Since the bark extracts used in experiments 2 and 3 were crude, rather than purified tannins, it is possible that another chemical within the extract could be affecting the diversity and growth rates of the fungi.

Despite condensed tannin discrepancies my total tannin concentrations did match previous literature. The total tannin concentrations 0.18g/8g sample in American chestnut vs. 0.26g/ 8g sample in Chinese chestnut) were similar to those found by Anagnostakis (1992) (0.07 – 0.14 g/8g sample in AC vs. 0.14 – 0.22 g/ 8g sample in CC).

My third prediction was that the condensed tannins of the American chestnut tree would inhibit the growth of many fungi, allowing the *C. parasitica* to compete more

easily. Although it had a number of methodological problems, described in the results, experiment 3 showed that 5 of 6 fungi that could be directly compared grew at a faster rate on medium containing Chinese chestnut extract than containing American chestnut medium. These results are consistent with my hypothesis, but further work is needed in order to ensure the results were not simply due to the one or more of the problems encountered.

Fungal competition

A fourth experiment also needs to be added to complete the whole picture. The experiments that have been completed look indirectly at what factors may affect fungal competition within each tree species. The next experiment should look directly at competition. To do this I would grow *C. parasitica* and another fungal isolate together on plates with extracts from each tree species and without tannins. I predict that if condensed tannins, or another compound within the bark, affect competition levels, then *C. parasitica* would be able to better compete with other fungi on American chestnut extracts than on extracts of Chinese chestnut or post oak.

Fungal growth rates

The fungi growing on the American chestnut were slower growing than those from other trees. Nearly all of these fungi had similar morphological characteristics, such as gray pigment and irregular shape. Based on their similar appearance, these slower growing fungi may all be closely related to each other. There may also be a relationship between the chemical content, particularly tannin, of the American chestnut trees and the presence of slow growing fungi. The production of tannase can vary between fungi species, and thus in the fungi's ability to effectively digest tannins. A study of tannase

production, similar to that done by Farris (1992) with *C. parasitica*, may reveal a tradeoff between the growth rate and the ability to produce tannase. Perhaps the slow growers have evolutionarily traded fast growth for high tannase production.

Microbial diversity

Based on the cumulative number of fungal species found (Figure 2) I do not believe I have found all of the fungi growing on these three tree species. A more exhaustive survey of the microorganisms may strengthen the differences found in microbial diversity. This survey should include more branch samples, trunk samples from varied heights, and possibly even the leaf samples. Previous studies have shown that trees have the potential of hosting many more species of fungi than I found (Müller and Hallaksela 2000, Kowalski and Kehr 1996).

I also only sampled trees growing in two locations per tree species. By increasing the diversity of habitats sampled (deep woods, open fields, slopes) and the number of sites per habitat, a wider range of microbes growing on each tree species may be found. Reed and McCarthy (1996) studied the tannin content of chestnut oak (*Quercus prinus* L.) and black oak (*Quercus velutina* L.) growing on north vs. south- facing slopes. They found that chestnut oaks had larger amounts of tannins in their leaves on north-facing slopes than on south-facing slopes but that the tannin content of the black oak was not slope dependent. Any chemical variation among trees that is related to habitat variation could also lead to a varied population of microorganisms among trees.

When I surveyed each tree species I used potato dextrose agar, which is a medium suited for growing fungi (R. Davies, personal communication). If I also used a medium with a fungal inhibitor I could select for bacteria and possibly find more bacteria growing

under the bark of each tree. Using a bacteria specific method may also provide further insight on the lower frequency of bacteria and yeast found on the American chestnut samples. After a larger bacteria sample size is established various characteristic tests (such as gram -/+, H_2S production, motility, carbohydrate fermentation and a litmus milk test) could be run to compare the bacteria that tend to be found on each tree species

Fungal identification

My fungal identification was based solely on the macroscopic morphological characteristics of each fungal isolate. I looked at the color, growth rate, and structural differences between the isolates. In my identification, if two fungi had similar characteristics, but not necessarily identical ones, they were considered the same species. However, it is possible that two similar fungi are simply closely related species. Taxonomic study of the isolates is necessary before the exact diversity of fungi found can be reported.

Tannin methods

Because the tannin content results differed from previously published papers, further investigation of the tannins within each tree should be carried out. It is possible a different tannin assay method would reveal more accurate results. Hagerman and Butler (1988) discussed the advantages and disadvantages of several different tannin assay methods. The radial diffusion method was recommended because of its ease, and because it is not affected by non-tannin compounds in the solution or by the solvent used for the extraction (Hagerman 1987, Hagerman and Butler 1988). This method was successfully used by Anagnostakis (1992) with purified tannin extracts and yielded similar total tannin results to my findings. The focus of a future study should be on

altering the methods for determining the total condensed versus hydrolyzable tannin content. A chemical method that assays for specific functional groups may give more accurate results. The vanillin assay described by Hagerman and Butler (1988) would select for the condensed tannins in a mixture of varied tannin types by specifying for the flavanol units of the condensed tannins. However, I do not know if there are any other flavanol compounds in my crude extracts that might interfere with the method. A purification of the tannins in my samples may be necessary to accurately determine their condensed and hydrolyzable tannin content.

I also would like to repeat the third experiment (growth rates of fungi with Chinese chestnut and American chestnut extract) and include medium with post oak extracts. When making the medium I would use 4% agar instead of 6%. Although 4% agar does not completely solidify in a short time I believe if it is allowed to dry for a day or two before use it will solidify.

Wider survey of trees

If, after further testing, my tannin results are reproducible, a wider survey of individual trees would be needed. Reed and McCarthy (1996) showed that habitat variation can affect the leaf tannin content of oak trees, and geographic intraspecific variation in plant allelochemicals has been well documented. A combined study with several researchers that looked at trees from varied habitats and geographic regions may provide a more accurate picture of the typical condensed and hydrolyzable tannin content in each tree species.

Other active anti-microbial chemicals

When I compared the growth rates of several fungi on media containing extracts from American and Chinese chestnut I used the crude extracts and not the purified tannins. If, after further testing, my tannin results are reproducible, then it is likely that tannins are not responsible for the differences in microorganisms among tree species. It is possible other chemicals within the tree bark are affecting the fungal growth and diversity. For example, Havir and Anagnostakis (1998) reported that peroxidases in American and Chinese chestnut bark [have been a recent compound of interest in the study of resistance because, peroxidases have been shown to inhibit fungal enzyme activity.

CONCLUSION

My experiments were designed to determine whether variation in condensed tannin content in American chestnuts, Chinese chestnuts, and post oaks affects the level of endophytic fungal competition within a tree; and ultimately whether this is correlated with the level of resistance to chestnut blight disease in each tree species. Conclusions on the varied level of tannins within each tree species cannot be drawn until the data are confirmed with alternate assay methods because my results did not match those previously published. However, I did find a statistical difference in the frequency of fungi found in each tree species and a possible difference between the numbers of species of fungi found in each species. There is also suggestive evidence that fungi grow much faster on Chinese chestnut extracts than American chestnut extracts and that the fungi on Chinese chestnut and post oak trees are faster growing in general than those found on American chestnuts. An increased number of fungi and rate of fungal growth found on

Chinese chestnut and post oak trees may inhibit chestnut blight's ability to establish a hold on these trees. Further research will need to be carried out before the effects of condensed tannins and fungal competition can be tied to the varied levels of resistance among chestnut and oak trees.

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